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Asymmetric reduction of 4-hydroxy-2-butanone to (*R*)-1,3-butanediol with absolute stereochemical selectivity by a newly isolated strain of *Pichia jadinii*

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Abstract In this study, a novel strain of *Pichia jadinii*, HBY61, capable of the biocatalysis of 4-hydroxy-2-butanone (4H2B) to (*R*)-1,3-BD was isolated. HBY61 produced (*R*)-1,3-BD with high activity and absolute stereochemical selectivity (100 % e.e). Glucose and beef extract were found to be the key factors governing the fermentation, and their optimal concentrations were determined to be 84.2 and 43.7 g/L, respectively. The optimal bioconversion conditions of 4H2B catalyzed by HBY61 were pH 7.4, 30 °C, and 250 rpm with 6 % (v/v) glucose as the co-substrate. Accordingly, when 45 g/L of 4H2B was divided into three equal parts and added successively into the system at set time intervals, the maximum (*R*)-1,3-BD concentration reached 38.3 g/L with high yield (85.1 %) and strict 100 % enantioselectivity. Compared with previously reported yields for the biocatalytic production of (*R*)-1,3-BD, the use of strain HBY61 provided a high yield with excellent stereoselectivity.

T. Yang and Z. Man contributed equally to this work.

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Keywords (*R*)-1,3-butanediol · 4-Hydroxy-2-butanone · Asymmetric reduction · Optimization · *Pichia jadinii*

Abbreviations

4H2B	4-Hydroxy-2-butanone
(*R*)-1,3-BD	(*R*)-1,3-butanediol
e.e.	Enantiomeric excess
PB	Plackett–Burman
CCD	Central composite design

Introduction

Optically active alcohols are useful for organic syntheses of chemical catalysts, liquid crystals, flavors, agrochemicals, and drugs [4–7, 25]. The production of single enantiomers of alcohols has become increasingly important in the pharmaceutical industry [15]. The simplest and most powerful way to prepare enantiomerically pure alcohols is the asymmetric hydrogenation of ketones [4, 31]. (*R*)-1,3-Butanediol [(*R*)-1,3-BD] is an important chiral synthon for the synthesis of various optically active compounds such as azetidinone derivatives, which are key intermediates for the production of penem and carbapenem antibiotics [24].

(*R*)-1,3-BD can be produced by either chemical synthesis or microbial fermentation. Known processes for producing optically active 1,3-BD include (1) optical resolution of a chemically synthesized mixture of 1,3-butanediol racemates with an optical resolving agent [21], (2) conversion of 4-hydroxy-2-butanone (4H2B) by asymmetric synthesis with a raney nickel catalyst treated with an optically active compound, and (3) conversion of 4H2B by stereo-specific microbial oxido-reduction based on enantioselective oxidation of the undesired (*S*)-1,3-BD in the racemate [21, 24, 34]. However, both

processes (1) and (2) require either an expensive optical resolving agent or catalyst while the product obtained by process (2) has a low optical purity. As an alternative to chemical processes, biocatalysis [process (3)] has many advantages such as mild reaction conditions, few by-products, high stereoselectivity, no residual metals in the products, and avoidance of protection and deprotection of functional groups during the bioreaction. Therefore, process (3) is more efficient and environmentally friendly. Our current work is based on the fundamentals of process (3), which is a promising method to produce (*R*)-1,3-BD through stereo-specific microbial oxido-reduction.

Many patents [8, 10, 20, 22, 23, 26, 30] and non-patent [9, 12, 24, 34] documents describe microbial oxido-reduction [process (3)], using a variety of species such as *Candida*, *Kluyveromyces*, *Hansenula*, *Issatchenkia*, *Pichia*, *Bacillus*, *Pseudomonas*, *Rhodococcus*, and engineered *Escherichia coli.* Matsuyama et al. achieved (*R*)-1,3-BD production on a 10-L jar fermenter scale with the optical purity of 93 % e.e. using *K. lactis* IFO 1267 [24] and 94 % e.e. using *C. utilis* IAM 4277 [23]. An optical purity of >99 % e.e was achieved by Zheng et al. [34] with *C. krusei* ZJB-09162. As indicated by these results, microorganisms belonging to the same species can show different catalytic properties depending on the strain.

In the present study, we report the isolation and characterization of a newly isolated yeast, which is capable of catalyzing 4H2B to (*R*)-1,3-BD with high activity and absolute stereochemical selectivity (100 % e.e.). This strain was identified and named as *Pichia jadinii* HBY61. Other *Pichia* species have been reported for this purpose including *P. cellokiesa*, *P. heedii*, *P. lindnerii*, *P. pastoris* [23, 24], but they showed only moderate optical purity (<90 % e.e.), while the use of *P. jadinii* as a biocatalyst for the synthesis of (*R*)-1,3-BD from 4H2B has not been reported. In this report, we discuss the effect of nutritional and physical parameters on the production of (*R*)-1,3-BD using conventional methods and statistical analysis. The optimized parameters are suitable as a starting point for adapting the method to the industrial production of (*R*)-1,3-BD from 4H2B by *P. jadinii* HBY61.

Materials and methods

Chemicals

4-Hydroxy-2-butanone, (*S*)-1,3-butanediol, and (*R*)-1,3-butanediol were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). All the other components of the culture media and the chemical reagents were obtained from commercial sources.

Strains and culture conditions

All microorganisms used for screening were type cultures preserved in our laboratories. The medium used for yeast incubation (YPD) and culture contained yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, and ampicillin 100 μg/mL at pH 6.0. The strains were cultivated at 30 °C and 160 rpm for 48 h and the cells were collected and washed with 100 mM potassium phosphate buffer (pH 7.0), and then used directly for the biotransformation without pretreatment. For the bioconversion of 4-hydroxy-2-butanone to (*R*)-1,3-BD, a reaction mixture comprising 100 mM potassium phosphate buffer (pH 7.0), 100 mg 4-hydroxy-2-butanone, 0.4 g glucose, 300 mg (dry cell weight) active cells, in a final volume of 10 mL, was incubated at 30 °C on a reciprocal shaker (200 rpm). Samples (1.0 mL each) were withdrawn at regular intervals and the reaction was quenched by the addition of 10 μL HCl (6 M). After centrifugation, 200 μL supernatant was saturated with sodium chloride and extracted with 1 mL ethyl acetate. The ethyl acetate layer was collected and dried over anhydrous Na_2SO_4 for GC analysis.

Shaking flask effects of fermentation were investigated in 500 mL Erlenmeyer flasks containing 100 mL of fermentation medium. The size of the inoculum was 5 % (v/v). The basic fermentation medium before optimization contained 20 g/L glucose, 10 g/L beef extract, and 0.2 g/L $MgSO_4 \cdot 7H_2O$. The cultures were incubated at 37 °C on a rotary shaker at 160 rpm.

Identification of strains

Cell morphology of the yeast was observed by a scanning electron microscope (Quanta 200 SEM, FEI, Eindhoven, Holland). The cells grown on YPD broth or agar for 1–3 days were used for microscopic analysis. Carbon source use was examined using a standardized micromethod, API 20 C AUX Test (BioMerieux SA, Etoile, France). Cupules showing turbidity significantly greater than that of the negative control were considered positive. Identification was made by generating a microcode and analysis achieved by BioMerieux ATB Expression (Etoile, France) equipped with the V3.0 database.

The isolated yeast strain was also taxonomically identified by determining the sequences of its 18S rRNA gene. The 18S rRNA gene sequence was amplified by PCR with primers Pf (5′-ACCGGAATTC GCCTGAGAAACGGCTACC-3′) and Pr (5′-ACCGGAATTCGGCAGGGACGTAATCAAC-3′). The design, synthesis, and purification of oligonucleotide primers were performed by Sangon Biotech Co., Ltd. (Shanghai, China). DNA isolation and polymerase chain reaction (PCR) amplification with the Pf and Pr primers were done as described by Kurtzman [17].

The sequence of the 18S rRNA gene fragment was determined by Sangon Biotech Co., Ltd. The 18S rRNA gene sequence of *P. jadinii* HBY61 was submitted to GenBank under accession number GU324552.1. Alignment of the 18S rRNA gene sequence was obtained by BLAST searching from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Kimura 2-Parameter Distance model, MEGA ver. 2.1 [14], was applied for the calculation of evolutionary distance, and a phylogenetic tree was constructed using the neighbor-joining method. Statistical experiment design and data analysis were applied to the calculation of evolutionary distance, and a phylogenetic tree was constructed using the neighbor-joining method.

Statistical experiment design and data analysis

A three-step experimental design based on statistical methods was used to optimize the medium for (*R*)-1,3-BD production. The Plackett–Burman (PB) design was first used to select significantly variables for 1,3-BD production. Following that, the steepest ascent was generated by the first-order empirical equation obtained by the PB design to move rapidly towards the neighborhood of the optimum response. The center point of the PB design was taken as the origin for the path of steepest ascent. The last step in the formulation of the medium was to determine the optimum levels of significant variables for (*R*)-1,3-BD production. For this purpose, response surface methodology (RSM) based on central composite design (CCD) with five coded levels was employed to determine the most significant factors screened by PB design for enhancing (*R*)-1,3-BD production. Theoretical details of PB and RSM design are described in the supplementary materials. Design-Expert, Version 7.0 (STAT-EASE Inc., Minneapolis, MN, USA) was used for the experimental designs and statistical analysis of the experimental data. Analysis of variance was used to estimate the statistical parameters.

Analytical procedures

Quantitative analysis of substrate and product was performed with a gas chromatograph apparatus (SHIMAZU GC-14B, FID-detector, 2 m × Ø5 mm stainless steel column packed with chromosorb101 and operated with N_2 as carrier gas at a flow rate of 40 mL/min, detector temperature 230 °C, and column temperature 225 °C) [24, 28]. The e.e. of the products was determined by HPLC with a Chiralcel OB-H (4.6 mm × 25 cm: Daicel Chemical Ltd., Tokyo, Japan) and detection performed at 220 nm. The mobile phase is a mixture of *n*-hexane and 2-propanol (85/15, v/v) at a flow rate of 0.5 mL/min. The cell dry weight was determined as described by Du et al. [3]. Results reported are the mean of determinations on duplicate independent samples.

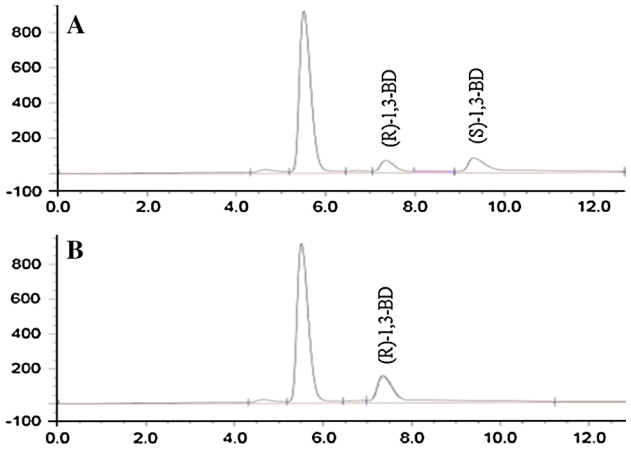

Fig. 1 Chiral analysis of the biotransformed sample. **a** Chiral analysis of the racemic 1,3-BD standard (the *left* peak appearing at 7.36 min is (*R*)-1,3-BD, and the *right* one appearing at 9.32 min is (*S*)-1,3-BD). **b** Chiral analysis of the biotransformation sample

Results

Screening of strains

All microorganisms used were type cultures preserved in our laboratories. The medium used for yeast incubation was also commonly used for yeast culture. Many microorganisms have been identified to produce optically active 1,3-BD from 4H2B [12, 24, 30, 34]. The isolate HBY61 was able to reduce 4H2B to (*R*)-1,3-BD with high activity and excellent stereoselectivity (100 % e.e.) (Fig. 1). Strain HBY61 was deposited in the China Center for Type Culture Collection (CCTCC M 2010335) for further studies.

Identification of strain HBY61

Cream-colored, convex, and smooth colonies with margins varying from smooth to lobed were produced on YPD agar. Microscopic observation of HBY61 grown on YPD broth showed that the cells were ovoidal to elongate in shape (Fig. 2). Carbon substrate assimilation profiles of the isolate using API 20 C AUX are summarized in Table 1. Strain HBY61 was then identified as *Pichia jadinii* [16] using ATB identification software by entering the seven-digit numerical profile.

The 18S rRNA gene sequence of the isolate HBY61 was determined, and a phylogenetic tree was constructed (Fig. 3). Strain HBY61 was closely clustered with *P. jadinii*

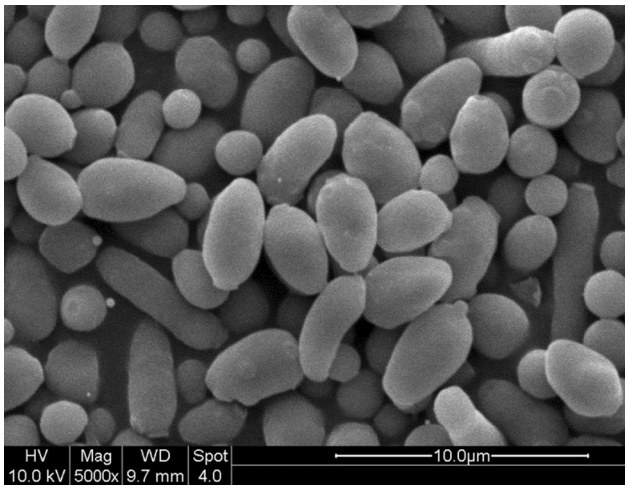

Fig. 2 Microscopic observation of strain HBY61

Table 1 Assimilation of carbon substrates by strain HBY61

Carbon substrate	HBY61	Carbon substrate	HBY61
D-Glucose	+	D-Trehalose	−
D-Maltose	+	Calcium 2-keto-gluconate	−
Sucrose	+	Inositol	+
D-Lactose	−	D-Melezitose	−
D-Galactose	+	Glycerol	+
D-Xylose	−	L-Arabinose	−
D-Cellobiose	−	Melibiose	−
D-Raffinose	+	D-Sorbitol	+

NRRL Y-1542 (GenBank accession no. EF550447.1), having a sequence identity of 99 %. Based on the results of phylogenetic analysis and phenotypic tests, the isolate was designated as *P. jadinii* strain HBY61.

Optimization of medium composition

PB design for a total of eight variables was used to identify which variables have significant effects on (*R*)-1,3-BD production (Table 2). The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via a Student's *t* test for analysis of variance (Table S1). Factors evidencing *P* values of less than 0.05 were considered to have significant effects on the response and were, therefore, selected for further optimization studies. Beef extract, with a probability value of 0.0046, was determined to be the most significant factor, followed by glucose (0.0168). To search the proper direction of these two factors with the other factors fixed at zero level, the path of the steepest ascent was employed. The design and responses of the steepest ascent experiment are shown in Table 3. The highest response was 91.2 % when the concentration of beef extract and glucose was selected to be 40 and 80 g/L, respectively. This suggested that this point was near the optimum and this combination was used as the middle point for the second-order experiment, i.e., Central composite design (CCD). The central points chosen for the experimental design were 40 g/L beef extract and 80 g/L glucose. The CCD design and the corresponding experimental responses are listed in Table 4. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following quadratic equation:

$$Y = 9.53 + 0.27X_1 + 0.47X_2 - 0.072X_1X_2 - 0.74X_1^2 - 0.77X_2^2,$$

where Y is the predicted (*R*)-1,3-BD production (g/L); X_1 and X_2 are the coded values of glucose and beef extract, respectively.

On the basis of the experimental values, statistical testing was carried out using Fisher's test for the analysis of variance (Table S2). A *P* value below 0.05 indicates that the model terms are significant. In this case, beef extract and glucose had significant effects on 2,3-BD production ($P < 0.05$). The fitness of the model was checked by the coefficient of determination, R^2, which was calculated to be 0.9911, showing that 99.11 % of variability in the response could be explained by the model. The value of the adjusted determination coefficient (Adj $R^2 = 98.48$ %) was also very high, confirming the high significance of the model. The R^2 value of this model was higher than 0.9, also indicating it had a very high correlation. Thus, it was reasonable to use the regression model to analyze the trends in the responses [13].

The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The effects of beef extract and glucose on (*R*)-1,3-BD production were also checked by the 3D response surface and 2D contour plots (Fig. 4). The model predicted the optimal values of the two most significant variables were $X_1 = 0.21$ and $X_2 = 0.37$. Correspondingly, the values of beef extract and glucose were 84.2 and 43.7 g/L, respectively. The maximum predicted value of (*R*)-1,3-BD was 93.5 %.

Based on the results of medium optimization, the optimum composition for (*R*)-1,3-BD production by *P. jadinii* HBY61 is as follows (g/L): glucose 84.2, beef extract 43.7, sodium citrate 3, $MgSO_4 \cdot 7H_2O$ 0.3, $ZnSO_4 \cdot 7H_2O$ 0.06, $FeSO_4 \cdot 7H_2O$ 0.06, $NaH_2PO_4 \cdot 12H_2O$ 2, and $K_2HPO_4 \cdot 3H_2O$ 2. To validate the adequacy of the model equation for predicting maximum (*R*)-1,3-BD production, three additional experiments in shake flasks were performed using the predicted culture conditions. Under the optimized condition, the (*R*)-1,3-BD average yield was 96.7 %, which was in good agreement with the model predicted maximum value.

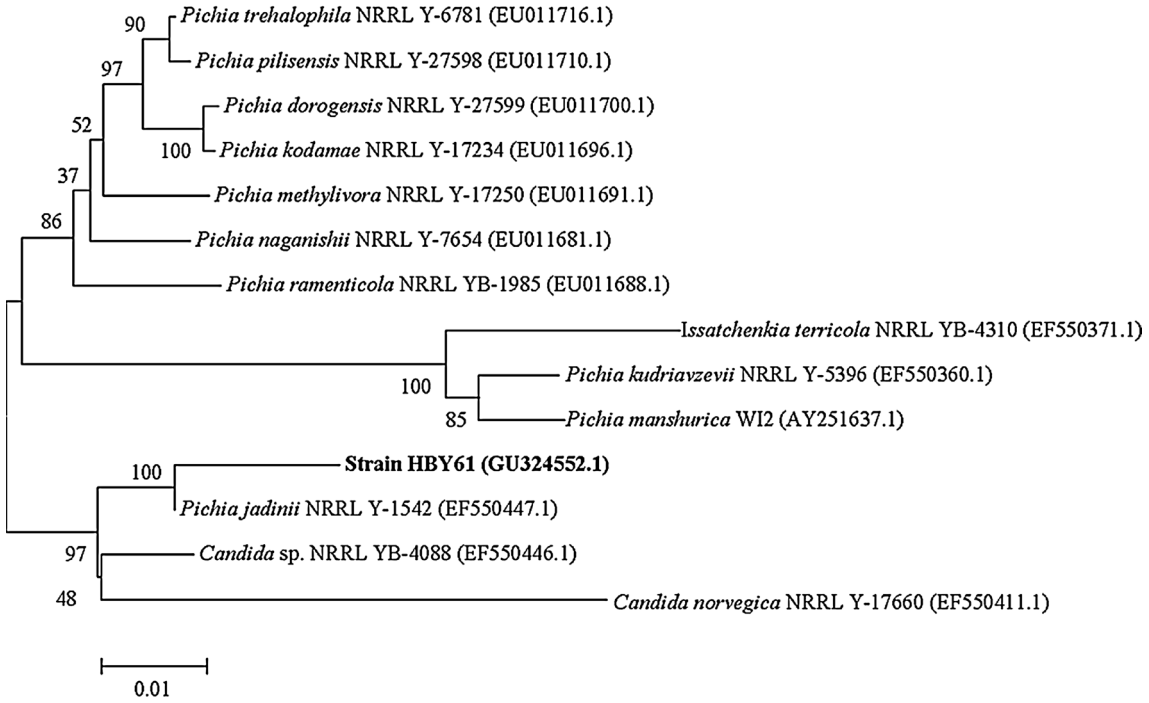

Fig. 3 The phylogenetic tree based on 18S rDNA sequences constructed by the neighbor-joining method shows the relationship between strain HBY61 and representatives of some related taxa. *Numbers in parentheses* are accession numbers of published sequences

Table 2 Plackett–Burman design for screening of significant factors affecting (*R*)-1,3-BD production

Run	Variable levels								Yield (%)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	
1	1 (8 g/L)	1 (80 g/L)	1 (30 g/L)	−1 (1 g/L)	−1 (0.02 g/L)	−1 (0.05 g/L)	1 (0.1 g/L)	−1 (1 g/L)	85.9
2	−1 (2 g/L)	1	1	−1	1 (0.1 g/L)	1 (0.2 g/L)	1 (0.02 g/L)	−1	80.7
3	−1	−1 (20 g/L)	−1 (10 g/L)	−1	−1	−1	−1	−1	61.5
4	−1	1	−1	1 (4 g/L)	1	−1	1	1 (4 g/L)	69.4
5	−1	−1	1	−1	1	1	−1	1	74.5
6	−1	1	1	1	−1	−1	−1	1	82.6
7	1	−1	1	1	1	−1	−1	−1	76.7
8	1	−1	1	1	−1	1	1	1	68.2
9	−1	−1	−1	1	−1	1	1	−1	59.4
10	1	1	−1	1	1	1	−1	−1	66.5
11	1	−1	−1	−1	1	−1	1	1	67.3
12	1	1	−1	−1	−1	1	−1	1	69.6

X_1 sodium citrate, X_2 glucose, X_3 beef extract, X_4 $NaH_2PO_4{\cdot}12H_2O$, X_5 $FeSO_4{\cdot}7H_2O$, X_6 $MgSO_4{\cdot}7H_2O$, X_7 $ZnSO_4{\cdot}7H_2O$, X_8 $K_2HPO_4{\cdot}3H_2O$

Effect of co-substrates on the asymmetric reduction of 4H2B to (*R*)-1,3-BD

Generally, carbonyl reductases require NADH or NADPH as a cofactor for catalyzing a reduction reaction. When whole microbial cells, such as yeast, are used as the catalytic system for the asymmetric reduction of carbonyl compounds, two enzymes can be involved in the

Table 3 Experimental design and results of the steepest ascent

Run	Glucose (g/L)	Yeast extract (g/L)	Yield (%)
1	40	20	62.5
2	60	30	73.7
3	80	40	91.2
4	100	50	80.9
5	120	60	72.2

Table 4 The results of the central composition experiment

Run	Coded variable level		Real variable level		Yield (%)
	X_1	X_2	Glucose (g/L)	Yeast extract (g/L)	
1	−1	−1	60	30	72.1
2	+1	−1	100	30	78.9
3	−1	+1	60	50	81.8
4	+1	+1	100	50	85.7
5	−1.414	0	71.72	40	77.3
6	+1.414	0	88.28	40	85.2
7	0	−1.414	80	35.86	73.1
8	0	+1.414	80	44.14	88.1
9	0	0	80	40	92.5
10	0	0	80	40	92.7
11	0	0	80	40	91.3
12	0	0	80	40	92.1
13	0	0	80	40	91.9

production reaction. One is the enzyme catalyzing the asymmetric reduction of prochiral carbonyl compounds to chiral alcohols, i.e., the carbonyl reductase. The other is a cofactor regeneration system, which supplies NADH or NADPH through the oxidation of the energy source, such as carbohydrates and alcohols [11]. In whole cells, the NADPH or NADH is successfully regenerated from intracellular metabolism of easily available carbohydrates or alcohols as the substrate, thus avoiding supplementation of additional expensive NADH or NADPH. Therefore, 4H2B (10 g/L) was reduced by *P. jadinii* HBY61 in the presence of methanol, ethanol, 2-propanol, 1-butanol, glucose, sucrose, or glycerol as co-substrates as a way to regenerate NAD(P)H. The results are shown in Fig. 5a. The yields were increased from 21.6 to 31.1, 54.5, 48.8, 55.7, 98.6, 86.9, and 72.3 % when the optimum concentrations of methanol, ethanol, 2-propanol, 1-butanol, glucose, sucrose, and glycerol were 4 % (v/v), 5 % (v/v), 2 % (v/v), 1 % (v/v), 5 % (w/v), 5 % (w/v), and 5 % (v/v), respectively. When the bioconversion is performed without co-substrate addition, the reduction reaction can only use NAD(P)H accumulated during yeast cell cultivation, which limits the yield to about 21 % (w/w). When a co-substrate such as glucose is added, the alcohol dehydrogenase inside the yeast cell can regenerate NAD(P)H, thereby increasing the yield. However, if the concentration of co-substrate is too high, it can inhibit the reduction process, which lowers the yield. The co-substrate can also change the permeability of the cell wall, which will help transport of the primary substrate into the cell. A concentration of 5 % (w/v) glucose was selected as a co-substrate for further optimization.

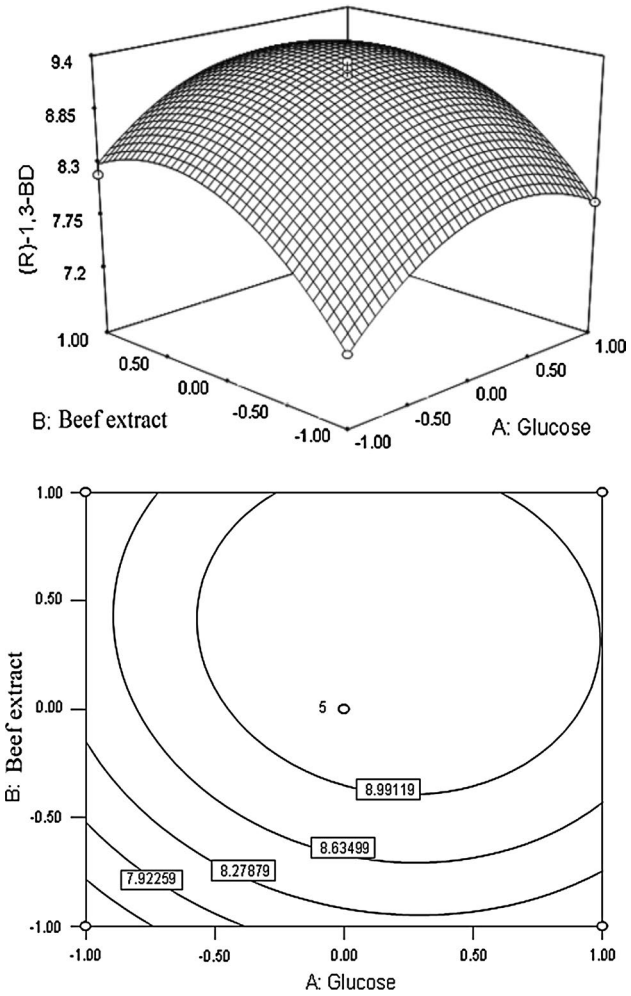

Fig. 4 Response surface figure and corresponding contour of the mutual effects of glucose and beef extract on the asymmetric reduction of 4H2B to (*R*)-1,3-BD

Effect of reaction temperature on the asymmetric reduction of 4H2B to (*R*)-1,3-BD

The efficiency of bioprocesses is highly dependent on temperature because of the variability of enzymatic activity and cellular maintenance as temperature changes. The effect of temperature on the asymmetric reduction of 4H2B to (*R*)-1,3-BD was investigated in the standard reaction system at temperatures varying from 20 to 45 °C while keeping the rest of the operating conditions constant. As shown in Fig. 5b, when the temperature was adjusted from 20 to 30 °C, the product yield increased slightly. Further increases in temperature led to a drastic drop in the product yield as the deactivation of the enzyme was accelerated at higher temperatures. The best yield was observed at 30 °C. The enantiometric excess of (*R*)-1,3-BD remained 100 % e.e. regardless of the temperature.

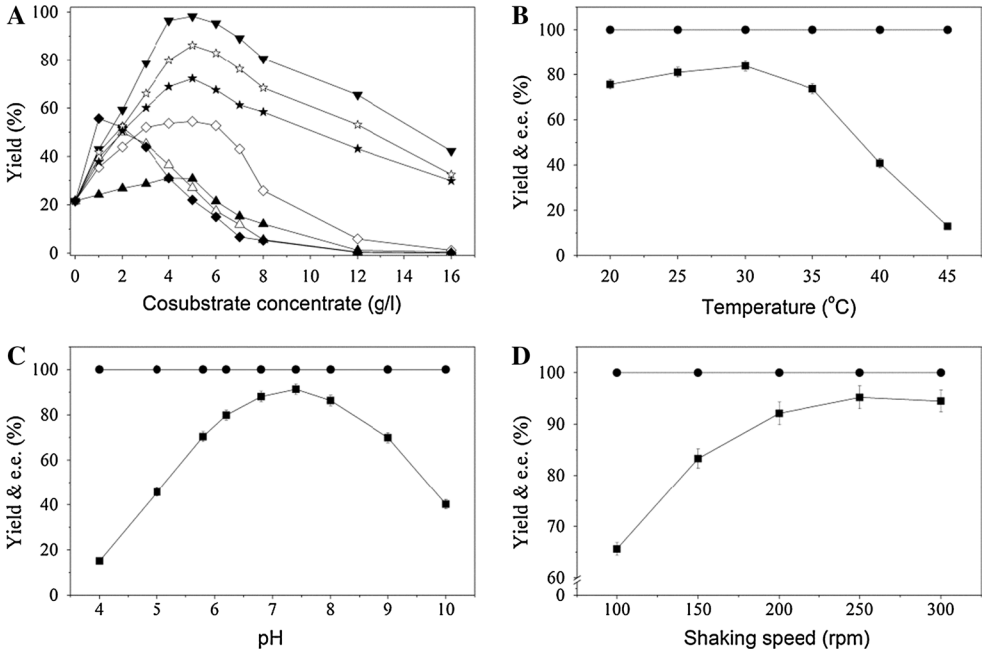

Fig. 5 Effect of co-substrates and culture conditions on the asymmetric reduction of 4H2B to (*R*)-1,3-BD. **a** Effect of co-substrates on (*R*)-1,3-BD production; **b** effect of temperature on (*R*)-1,3-BD production; **c** Effect of pH on (*R*)-1,3-BD production; **d** effect of the shaking speed on (*R*)-1,3-BD production. Methanol (*filled triangle*), ethanol (*open diamond*), 2-propanol (*open triangle*), 1-butanol (*filled diamond*), glucose (*inverted filled triangle*), sucrose (*open star*) and glycerol (*filled star*), Yield (*filled square*), e.e. (*filled circle*)

Effect of the pH of the potassium phosphate buffer on the asymmetric reduction of 4H2B to (*R*)-1,3-BD

Enzymes are polyelectrolytes containing both positive and negative ionizations of amino acids at the surface, which can be protonated or unprotonated depending on the pH of the surrounding medium. Thus, the effect of pH in the water phase localized around active cells is an important consideration. There is a pH range for each enzyme at which the conformation of the enzyme is optimal for catalytic activity, while extreme pH conditions will cause an irreversible inactivation of the enzyme.

To determine the optimum pH of the biocatalytic asymmetric reduction of 4H2B, the reaction was carried out at 30 °C in media at different pHs varying from 4.0 to 10.0 [acetate buffer (pH 4–6), potassium phosphate buffer (pH 6–8), Tris–HCl buffer (pH 8–9), and carbonate buffer (pH 9–10)]. As shown in (Fig. 5c), *P. jadinii* HBY61 has great reduction ability from pH 5.8 to 9.0 with an optimum at pH 7.4. pH values outside this range were detrimental to the bioconversion, especially at acidic pHs (pH < 5). When the pH value dropped to 4, the yield sharply decreased from 45.7 to 15.1 %. Thus, pH greatly influenced the product yield, which presumably was a result of activity changes of key enzymes at extremes of pH. The enantiometric excess of (*R*)-1,3-BD was 100 % regardless of the pH of the media.

Effect of shaking speed on the asymmetric reduction of 4H2B to (*R*)-1,3-BD

Rotating speed influences the diffusion and partition of the substrate and product in the reaction system and thus can impact the yield [31]. The effect of shaking speed (100–300 rpm) on the asymmetric reduction was investigated (Fig. 5d). It was found that the product yield increased when the shaking speed was below 250 rpm, indicating that mass transfer was the rate-limiting step. The optimal shaking rate was shown to be 250 rpm, above which no obvious increase in the yield was observed. The e.e. of (*R*)-1,3-BD was maintained at 100 % at all shaking speeds tested.

Preparative scale conversion of 4H2B to (*R*)-1,3-BD

Under the optimized conditions, a preparative scale (200 mL) asymmetric reduction of 4H2B was carried out using *P. jadinii* HBY61. As shown in Fig. 6, when 45 g/L of 4H2B was added in the reaction buffer at one time, the titer of (*R*)-1,3-BD and yield were only 27.1 g/L and 60.2 % at 60 h with a (*R*)-1,3-BD productivity of 0.38 g/L h. This was likely a result of the fact that a large amount of substrate is toxic to the microorganism and inhibits the activity of the enzyme. To reduce the substrate inhibition, the substrate was divided into three equal parts and added one at a time into the system every 20 h (Fig. 6). It was obvious that when the substrate were divided into three equal parts, the titer and yield of (*R*)-1,3-BD were 38.3 g/L and 85.1 % at 60 h with a (*R*)-1,3-BD productivity of 0.53 g/L h.

To determine the scale-up capacity, biocatalysis was carried out using a 10-L fermenter with working volumes of 6 L. Optimal media and culture conditions determined by flask experiments were used for the scale-up tests. The cellular growth in the fermenter was higher than that in the flasks. In the fermenter, when the substrate was divided into three equal parts, the titer and yield of (*R*)-1,3-BD were

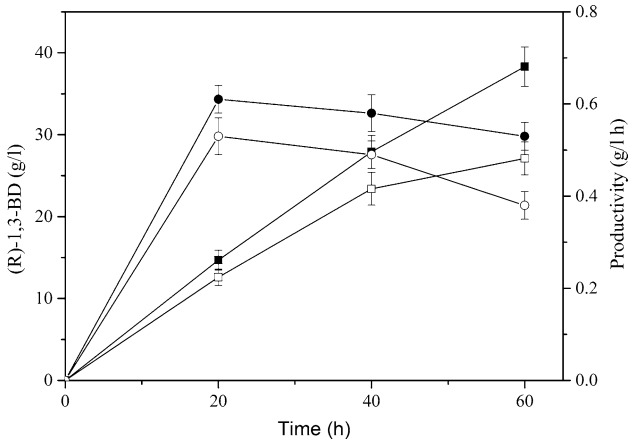

Fig. 6 Preparative scale conversion of 4H2B to (*R*)-1,3-BD. Titer of (*R*)-1,3-BD (addition of substrate in one batch *open square*, addition of substrate in three equal batches *filled square*), productivity (addition of substrate in one batch *open circle*, addition of substrate in three equal batches *filled circle*)

40.5 g/L and 90.0 % at 60 h with a (*R*)-1,3-BD productivity of 0.68 g/L h, which were higher than that obtained from the flasks.

Discussion

A novel strain of *P. jadinii*, HBY61, proved to be a good biocatalyst for the conversion of 4H2B to (*R*)-1,3-BD with high activity and absolute stereochemical selectivity (100 % e.e). This was an improvement of the stereochemical selectivity reported for strain *C. krusei* ZJB-09162 by Zheng et al. [34]. Furthermore, it has better selectivity than those reported for several *Pichia* species including *P. cellokiesa* DSM 2147 (79 % e.e.), *P. heedii* IFO 10019 (86 % e.e.), *P. lindnerii* DSM 70718 (78 % e.e.), *P. opuntiae var. thermotolerans* IFO 10025 (94 e.e.), *P. pastoris* DSM 70382 (87 % e.e.), *P. trehalophia* DSM 70391 (83 % e.e.) [23], and *P. heedii* IFO 10020 (81 % e.e.) [24]. Thus, *P. jadinii* HBY61 is a novel R-stereospecific carbonyl-reductase-producing yeast for the synthesis of (*R*)-1,3-BD from 4H2B.

Cell growth conditions and the production of the carbonyl reductase are strongly influenced by medium composition such as the carbon source, nitrogen source, and inorganic salt content. A more efficient analytical technique based on RSM [2] was used for optimization of media composition. Glucose and beef extract were found to be key factors in the fermentation yield. After optimizing their concentrations via a response surface methodological approach, they were determined to be 84.2 and 43.7 g/L glucose and beef extract, respectively.

Reduction of substrates usually requires large amounts of energy. For the majority of redox enzymes, NAD(P)H are essential endogenous coenzymes [33]. The main obstacle associated with the oxidoreductase catalyzed bioreduction from 4H2B to (*R*)-1,3-BD is the requirement for the expensive coenzyme NADPH. Because it is only the oxidation state of the coenzyme that changes during the reaction, it can be regenerated using a second redox-reaction to allow it to re-enter the reaction cycle [33]. Usually, formate [29], ethanol [18], 2-propanol [19], and glucose [32] are used to regenerate the oxidized form of NAD(P)H to the reduced form. The influence of co-substrates on 4H2B reduction with *P. jadinii* HBY61 was investigated and glucose was found to be the best co-substrate with an optimum concentration of 6 % (w/v). With the use of active cells of *P. jadinii* HBY61 as a biocatalyst, the NADPH was successfully regenerated from intracellular glucose metabolism with glucose as the substrate, thus avoiding supplementation of additional glucose dehydrogenase (GDH) and NADPH.

Asymmetric biocatalytic reduction was optimized in terms of several different reaction parameters, such as temperature, buffer pH, and shaking speed. Temperature has long been known to influence the activity, enantioselectivity, and stability of biocatalysts [27]. The effect, which is a consequence of the process of reaction acceleration because of increased temperature, is completely counter balanced at higher temperatures owing to thermal inactivation of the reaction enzymes [1]. Variation of buffer pH could alter the ionic state of the substrates and affect the local polarity of the enzyme's active site, thus, leading to changes in the enzyme's activity and enantioselectivity. The results indicated that the key enzymes were very sensitive to low and high extremes of pH. No variation of the enantiomeric selectivity of the product was observed within the assayed range of buffer pH and temperature. Shaking speed influences the diffusion and mass transfer of the substrate and product in the reaction system. Tests carried out at variable agitation speed demonstrated that external diffusion was a key rate-limiting factor when the agitation speed was lower than 250 rpm but it had little effect when above 250 rpm for the production of (*R*)-1,3-BD. The product e.e. was not been affected by the agitation speed.

To reduce the substrate inhibition, the substrate was added in equal parts such that the titer of (*R*)-1,3-BD and yield were 38.3 g/L and 85.1 % at 60 h with a (*R*)-1,3-BD productivity of 0.53 g/L h and e.e. of 100 %. Compared with the use of other microorganisms for the asymmetric reduction of 4H2B [9, 24, 34], the use of HBY61 is comparable in terms of the overall efficiency of the biocatalytic reaction (30–40 g/L). Furthermore, a current shortage of fossil fuel resources and petrochemical supplies make the bio-based production of 1,3-BD, especially with a high optical purity of (*R*)-1,3-BD, from renewable biomass an attractive alternative [12]. Recently, a synthetic metabolic route of (*R*)-1,3-BD production from glucose by the

recombinant *Escherichia coli* strain HB101 was reported by Okabayashi et al. [26], but a lower yield of 1.028 g/L with a moderate 86.6 % e.e. was obtained. To improve the optical purity and yield of (*R*)-1,3-BD, Kataoka et al. [12] constructed an effective synthetic biological production route of 1,3-BD from glucose in *E. coli* MG1655 lacI^q. At the optimized fermentation conditions, this recombinant whole-cell biocatalyst could produce up to 9.05 g/L 1,3-BD in 98.5 % e.e. of (*R*)-1,3-BD. Construction of a recombinant version of the *P. jadinii* HBY61 ketoreductase responsible for producing 1,3-BD with high e.e. from a fermentation substrate warrants further investigation as a way to improve the yield.

In conclusion, a new whole cell biocatalyst, *P. jadinii* HBY61, was used for the enantioselective reduction of 4H2B to (*R*)-1,3-BD for the first time. The biocatalyst activity was affected by the medium composition and conditions. Accordingly, when 45 g/L of 4H2B was divided into three equal parts and added sequentially to the system at given time intervals, the maximum (*R*)-1,3-BD concentration reached 38.3 g/L with strict enantioselectivity (100 % e.e.). The yields were relatively high compared with previously reported values. In addition, the excellent stereochemical selectivity of the new biocatalyst for (*R*)-1,3-BD was strict. The high reaction rate and its absolute stereochemical selectivity made *P. jadinii* HBY61 a promising biocatalyst for the asymmetric synthesis of (*R*)-1,3-BD for large-scale applications. Future work will include the isolation of the ketoreductase from *P. jadinii* HBY61 followed by engineering this enzyme to enhance its catalytic activity for the reduction of 4H2B for the asymmetric synthesis of (*R*)-1,3-BD, an important pharmaceutical intermediate.

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